Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 35-52 are pending in the application, with claims 35, 43, 46 and 49 being the independent claims. Claim 33 is sought to be cancelled without prejudice to or disclaimer of the subject matter therein. New claims 35-52 are sought to be added. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

I. Support for New Claims

Support for new claims 35-52 can be found throughout the specification, for example, at page 7, lines 11-13, at page 25, line 8, through page 33, line 3, and in original claim 33.

II. Claim Rejection Under 35 U.S.C. § 112, Second Paragraph

Claim 33 was rejected under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention. *See* Paper No. 12, page 2. The basis for this rejection is the phrase "with the a region" found in claim 33. Claim 33 has been canceled. None of

the newly added claims include the phrase at issue. Accordingly, the rejection under 35 U.S.C. § 112, second paragraph, is moot and should be withdrawn.

III. Claim Rejection Under 35 U.S.C. § 112, First Paragraph

Claim 33 was rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the enablement requirement. *See* Paper No. 12, page 2. According to the Examiner, the claim contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. *See* Paper No. 12, page 2. Applicants respectfully traverse this rejection.

Claim 33 has been cancelled. New claims 35-52 encompass subject matter which was encompassed by claim 33. Applicants submit that the subject matter of new claims 35-52 is fully enabled and that the rejection under 35 U.S.C. § 112, first paragraph, cannot properly be applied to the new claims.

The present invention is directed, in general, to the treatment or prevention of neuroectodermal tumors, malignant astrocytomas, or glioblastomas by interfering with AD7c-NTP expression at the level of transcription and/or translation. More specifically, the methods of the invention comprise administering to an animal in need thereof:

- an antisense oligonucleotide (claims 35-42);
- a ribozyme (claims 43-45);
- an oligonucleotide that forms one or more triple-stranded regions with the coding region of AD7c-NTP DNA (claims 46-48); and
- a ribonucleotide external guide nucleic acid molecule (claims 49-52).

The molecules used in the methods of the invention include nucleotide sequences that are complementary to or that correspond to nucleotides 150-1139 of SEQ ID NO:1.

The specification, along with the information available in the art, would have provided ample guidance for a skilled artisan to practice the presently claimed methods without undue experimentation. General information regarding the use of antisense oligonucleotides, ribozymes, triple helix-forming oligonucleotides, and ribonucleotide external guide nucleic acid molecules, for therapeutic purposes, is provided in the specification at page 28, line 20, through page 33, line 3. Additional information on the therapeutic use of these molecules would have been available to persons of ordinary skill in the art. Details regarding the appropriate route(s) of administration, methods for enhancing the cellular uptake of the molecules used in the methods of the invention, and the preparation of pharmaceutical formulations for use with the methods of the invention, would have been known by persons of ordinary skill in the art at the time of the effective filing date of the application. *See* Specification at page 30, line 14 through page 33, line 3. Thus, the practice of the claimed methods would not have required undue experimentation.

In order to establish a *prima facie* case of lack of enablement, the Examiner has the initial burden to set forth a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). To satisfy this burden, "it is incumbent upon the Patent Office. . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971) (emphasis in original). Applicants respectfully submit that the reasons set forth in

support of the rejection under § 112, first paragraph, are legally insufficient to establish a *prima facie* case of non-enablement.

According to the Examiner, "[t]he instant specification does not provide any specific guidance such as what particular antisense, ribozyme, external guide sequence, or tiplex forming oligonucleotide sequences could be used effectively in the claimed method." Paper No. 12, page 3. Applicants respectfully disagree. The specification sets forth the nucleic acid sequence of AD7c-NTP (SEQ ID NO:1). The claims specify that the molecules used in the practice of the methods are complementary to or correspond to nucleotides 150-1139 of SEQ ID NO:1. A skilled artisan would appreciate that antisense oligonucleotides, ribozymes, triple helix-forming oligonucleotides and ribonucleotide external guide nucleic acid molecules interfere with gene expression by recognizing complementary or corresponding nucleotide sequences and thereby inhibit transcription and/or translation. Based on the sequence information in the specification, and an understanding of the mechanisms by which antisense oligonucleotides, ribozymes, triple helix-forming oligonucleotides and ribonucleotide external guide nucleic acid molecules inhibit gene expression, a person of ordinary skill in the art would have been able to select the appropriate nucleotide sequences to effectively interfere with the expression of AD7c-NTP in an animal using the claimed methods.

The Examiner stated that "[t]he instant specification does not provide guidance or examples that would show by correlation what modes of delivery would predictable [sic] provide for a treatment of disease in general and for the treatment or prevention of neuroectodermal tumors, malignant astrocytomas and glioblastomas in particular." Paper No. 12, pages 3-4. Applicants respectfully disagree. The specification makes it clear that

AD7c-NTP overexpression *in the brain* is associated with Alzheimer's disease. *See, e.g.*, Specification at page 41, lines 18-28 (showing significantly higher levels of AD7c-NTP mRNA in AD brains versus aged control brains). A person of ordinary skill in the art would also understand that neuroectodermal tumors, malignant astrocytomas and glioblastomas are abnormalities associated with *neuronal tissue*. According to the specification:

> The NTP antisense oligonucleotide, NTP triple helix-forming oligonucleotide, NTP ribozyme or NTP EGS [external guide sequence], and the pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intra-peritoneal, transdermal, intrathecal or intracranial routes.

Specification at page 30, line 28, through page 31, line 3. Thus, a person of ordinary skill in the art would recognize that any mode of delivery that brings the compounds into contact with neuronal cells in an animal would be effective in the context of the present invention.

The Examiner also stated that: "[t]he instant specification does not provide any examples of inhibiting AD7c-NTP in cells in culture or in an animal or provide guidance that would show by correlation the treatment or prevention of neuroectodermal tumors, malignant astrocytomas and glioblastomas via the administration of antisense based nucleic acid compounds." Paper No. 12, page 4. Applicants submit that the technological field of inhibiting gene expression by use of antisense oligonucleotides, ribozymes, triple helix-forming oligonucleotides and ribonucleotide external guide nucleic acid molecules, was sufficiently advanced at the time of the effective filing date of the application, and the mechanisms by which such molecules functioned was well established. Therefore, a person of ordinary skill in the art would have been able to practice the methods of the invention without undue experimentation, even in the absence of a working example.

There are many examples from the scientific literature that demonstrate successful therapeutic applications of the kinds of molecules that are used in the practice of the claimed methods. For example, several instances of the successful application of antisense molecules *in vivo* are described in Galderisi *et al.*, *J. Cell. Physiol. 181*:251-257 (1999) (copy attached hereto as Exhibit A). Among the examples provided in Galderisi are the following:

- Antisense oligonucleotides against protein kinase C alpha (PCK-α) mRNA produced clinical responses when administered to ovarian cancer patients. *See* Galderisi at page 253, bottom right column;
- Antisense oligonucleotides against c-raf mRNA produced "promising clinical response[s]" in patients with breast, prostate, and colon cancer. *See* Galderisi at page 254, top left column;
- Antisense oligonucleotides against bcr/abl mRNA, when administered to a patient with chronic myelogenous leukemia (CML), resulted in "complete hematological remission." *See* Galderisi at page 254, middle left column;
- Antisense oligonucleotides targeted against c-myc mRNA, when delivered into balloon-denuded porcine coronary arteries, caused a reduction in neointimal thickness (which is usually increased following balloon angioplasty). *See* Galderisi at page 254, right column; and
- Antisense oligonucleotides directed against the 5'-region of the preS gene of duck hepatitis B virus (DHBV), injected intravenously into DHBV-infected ducks, inhibited DHBV replication and caused a decrease in serum DHBV DNA levels. *See* Galderisi at page 255, left column.

In addition, Agrawal, *Tibtech. 14*:376-387 (1996) (cited by the Examiner at page 4 of the Office Action) states that "many reports have appeared in the literature confirming the application of antisense technology in *in vivo* models." Agrawal at page 376, bottom left column. Agrawal goes on to describe eight specific examples where antisense technology has been successfully applied in animals. *See* Agrawal at page 376, paragraph bridging left and right columns.

The above-described examples support the enablement of the invention in two respects. First, the general techniques used in these examples were available as of the effective filing date of the application. In order to satisfy the enablement requirement of 35 U.S.C. § 112, first paragraph, an Applicant need not supply information that is known in the art. *See Genentech, Inc. v. Novo Nordisk*, 108 F.3d 1361, 1366, 42 USPQ2d 1001, 1005 (Fed. Cir. 1997). The examples set forth above, therefore, would have supplemented the teachings of the specification and would have provided additional guidance to those of ordinary skill in the art in practicing the currently claimed methods.

Second, the examples clearly demonstrate that antisense technologies, in general, can be used to successfully disrupt gene expression and can provide positive clinical outcomes. Since the antisense techniques used in the examples set forth above were effective, there is no reason to believe that such techniques would not be equally effective when used in the context of the present invention, *i.e.*, in targeting/disrupting AD7c-NTP expression.

The Examiner has cited three references that discuss various technical considerations related to the use of antisense molecules. Agrawal, *Tibtech. 14*:376-387 (1996) is cited for the proposition that "[o]ligonucleotide must be taken up by cells in order to be effective." Paper No. 12, page 4 (quoting Agrawal at page 378, bottom left column). The specific

portions of Agrawal cited in the Office Action relate to the cellular uptake of oligonucleotides *in culture*. *See* Agrawal at page 378, bottom left column, through page 379, middle left column (under the heading "Cell culture system and target gene"). Agrawal concludes, however, that "[i]t is clear from some of the studies mentioned in this review and many other published reports that PS-oligonucleotides show more sequence-specific antisense activity in animal models than in cell culture experiments." Agrawal at page 384, middle right column. The present invention relates to the administration of antisense oligonucleotides, ribozymes, triple helix-forming oligonucleotides and ribonucleotide external guide nucleic acid molecules *to animals*, not to cells in culture. Thus, the concerns with cellular uptake of oligonucleotides in cell culture, as discussed in Agrawal, are irrelevant with respect to the claimed methods. Importantly, Agrawal indicates that antisense technology *is* effective in animals, thereby supporting Applicants' position that the claimed methods are fully enabled.

The Examiner cited Branch, *TIBS 23*:45-50 (1998), for issues relating to "non-antisense effects" and accessibility of oligonucleotides to target RNA. With respect to "non-antisense effects" it is important to recognize that Branch's comments relate to the ability of an antisense molecule to precisely recognize one specific target and none others. It is noted by Branch, however, that "both ODNs [antisense oligonucleotides] and bioengineered ribozymes can undeniably hit their intended targets." Branch at page 45, bottom right column. Branch also notes that, in the pharmaceutical context, non-antisense effects may be advantageous. *See* Branch at page 46, middle left column ("Phase III clinical trials of ISIS 2922, a phosphorothioate oligonucleotide (S-ODN) that induces both antisense and non-antisense effects, are also under way in patients with cytomegalovirus-associated

retinitis. It is hoped that this compound's diverse mechanisms of action will yield a single drug that provides many of the benefits of combination therapy." (internal citations omitted)). Most of the concerns about "non-antisense effects" discussed in Branch relate to non-antisense effects in research settings, not in therapeutic settings. *See* Branch at page 46, middle left column. Thus, the potential for "non-antisense effects" does not support the conclusion that the present invention is not enabled.

With respect to oligonucleotide accessibility, Branch simply indicates that not all oligonucleotides are equal in their ability to bind to a particular RNA target, and that screening may be needed to identify optimal sequences. In the context of the enablement requirement, experimentation, even complex experimentation, is not undue if the art typically engages in such experimentation. *See In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom., Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985); *see also Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. Screening for oligonucleotides with enhanced accessibility to a target would not be regarded as undue experimentation.

As noted by Branch, "[o]ne approach [to enhance specificity within cells] has been to deploy multiple antisense compounds, each directed against a different site in the same target RNA and thereby achieve annihilation by molecular triangulation." Branch at page 48, bottom right column. Branch also describes examples in which researchers identified effective antisense oligonucleotides by screening multiple candidate oligonucleotides. *See* Branch at page 49, left and center columns. In one example, 1938 oligonucleotides were screened to identify those that could bind to a 122 nucleotide RNA representing the 5' end

of β-globin mRNA. *See* Branch at page 49, left column. In another example, an antisense oligonucleotide that was able to reduce the level of *c-raf* kinase mRNA by more than five-fold was identified by screening 34 candidate oligonucleotides. *See* Branch at page 49, paragraph bridging left and center columns, and Fig. 3. It is therefore clear that persons of ordinary skill in the art typically engaged in screening to identify effective antisense oligonucleotides and that such screening would not have been regarded as undue experimentation.

The statement in Branch at page 49, right column, that "[i]t is not yet clear whether *in vitro* screening techniques . . . will identify ODNs that are effective *in vivo*," does not indicate that such screening techniques would necessarily be ineffective or that they would be regarded as undue experimentation. As noted by Branch, "[i]f tests of 50 molecules identify good candidates, tests of thousands of compounds should identify better ones." Branch at page 49, right column. There has been no evidence presented to suggest that screening thousands of antisense oligonucleotides would have been regarded as undue experimentation. Thus, Branch does not support the assertion that the present invention is not enabled.

Moreover, at the time of the effective filing date of the application, computerized modeling of mRNA structure would have been available to persons of ordinary skill in the art to assist in the identification of mRNA targets. *See, e.g.*, Jaroszewski *et al.*, *Antisense Res. Dev. 3*:339-348 (1993) (abstract submitted herewith as Exhibit B). The selection of target sequences for antisense molecules using computer-based methods is discussed in Galderisi:

> Modeling of the secondary structure of the target mRNA by computer software can be used for target selection of

> antisense molecules. Such a method carefully considers the potential folding pattern of a chosen mRNA as derived from its particular nucleotide sequence. After determining the free energy of a given secondary structure, the most probable folding structures are indicated, showing open loops and bulges that are accessible for oligonucleotides for efficient hybridization.

Galderisi at page 252, bottom left column. The use of such computerized methods would have enabled persons of ordinary skill in the art to identify accessible regions in AD7c-NTP mRNA and design corresponding oligonucleotides.

Finally, the Examiner cited Jen and Gewirtz, *Stem Cells 18*:307-319 (2000), as indicating that "progress needs to be made in the art," and outlining the "key challenges" to the field. *See* Paper No. 12, page 7. The need for progress, and the existence of "challenges," however, does not indicate that antisense-based methods would have required undue experimentation. Jen and Gewirtz does not support a finding of non-enablement.

In summary, there are many examples in the scientific literature showing the successful clinical use of antisense-based technologies in the treatment of various diseases and conditions. The methods used in these examples would have been available to persons of ordinary skill in the art at the time of the effective filing date of the present application and therefore would have supplemented the teachings in the specification to enable the practice of the claimed methods. The references cited in the Office Action, at best, highlight certain technical considerations to be addressed in practicing antisense-based methods. The references, however, do not demonstrate that the practice of the claimed methods would have required undue experimentation. Accordingly, Applicants respectfully request that the rejection under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

Conclusion

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Antisense Oligonucleotides as Therapeutic Agents

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Antisense oligonucleotides can block the expression of specific target genes involved in the development of human diseases. Therapeutic applications of antisense techniques are currently under investigation in many different fields. The use of antisense molecules to modify gene expression is variable in its efficacy and reliability, raising objections about their use as therapeutic agents. However, preliminary results of several clinical studies demonstrated the safety and to some extent the efficacy of antisense oligodeoxynucleotides (ODNs) in patients with malignant diseases. Clinical response was observed in some patients suffering from ovarian cancer who were treated with antisense targeted against the gene encoding for the protein kinase C-alpha. Some hematological diseases treated with antisense oligos targeted against the bcr/abl and the bcl2 mRNAs have shown promising clinical response. Antisense therapy has been useful in the treatment of cardiovascular disorders such as restenosis after angioplasty, vascular bypass graft occlusion, and transplant coronary vasculopathy. Antisense oligonucleotides also have shown promise as antiviral agents. Several investigators are performing trials with oligonucleotides targeted against the human immunodeficiency virus-1 (HIV-1) and hepatitis viruses. Phosphorothioate ODNs now have reached phase I and II in clinical trials for the treatment of cancer and viral infections, so far demonstrating an acceptable safety and pharmacokinetic profile for continuing their development. The new drug Vitravene, based on a phosphorothioate oligonucleotide designed to inhibit the human cytomegalovirus (CMV), promises that some substantial successes can be reached with the antisense technique. J. Cell. Physiol. 181:251–257, 1999. © 1999 Wiley-Liss, Inc.

The use of oligonucleotides as selective inhibitors of gene expression offers a rational approach for the prevention and treatment of some gene-mediated disorders. In the antisense approach, oligonucleotides block the expression of specific target genes involved in the development of the pathological processes. Therapeutic applications of antisense technique currently are under investigation in many different fields, including oncology, hematopathology, cardiovascular diseases, and infectious diseases (Agrawal and Iyer, 1995; Wagner, 1995; Agrawal, 1996; Crooke and Bennet, 1996; Bradbury, 1997; Wagner and Flanagan, 1997; Agrawal and Zhao, 1998).

Antisense oligodeoxynucleotides (ODNs) are short stretches of DNA (12–30 nucleotides) that are complementary to a target mRNA. The ODNs selectively hybridize to their complementary RNA by Watson-Crick base pairing rules. The translation of target mRNA is inhibited by an active and/or a passive mechanism when hybridization occurs between the ementary helices. Passive mechanism results from the hybridization between the mRNA and exogenous nucleotide sequence, which leads to duplex formation that prevents the ribosomal complex from reading the message (Fig. 1A). In the active mechanism, hybridization allows for binding of RNaseH, which destroys the RNA but leaves the DNA oligonucleotide intact to hybridize with yet another mRNA target (Fig. 1B; Wagner and Flanagan, 1997; Bradbury, 1997; Monia, 1997; Kronenwett and Haas, 1998b).

The concept of antisense technology is simple. However, the development of antisense oligonucleotides as broadly applicable therapeutic agents has been slow and difficult (Stein and Cheng, 1993; Stein and Krieg, 1994; Stein, 1995; Bradbury, 1997; Wagner and Flanagan, 1997; Romano et al., 1998a).

SELECTION OF A SPECIFIC AND EFFECTIVE ANTISENSE MOLECULE

Selection of target sequence

The selection of an appropriate target sequence is the first step in the process of drug development. In

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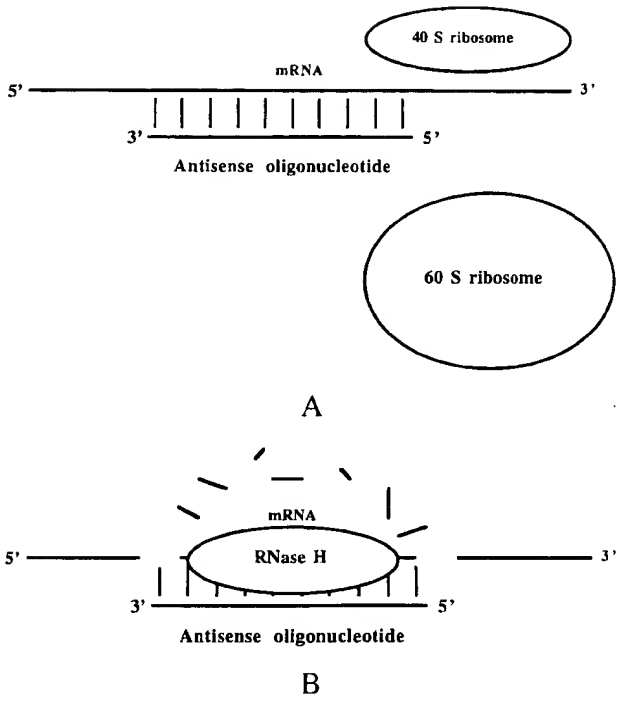

Fig. 1. Mechanism of action of antisense oligonucleotides. In the passive mechanism (**A**), the translation of target mRNA is inhibited by the hybridization between the mRNA and exogenous nucleotide sequence. This leads to duplex formation that prevents the ribosomal complex from reading along the message. In the active mechanism (**B**), the mRNA-ODN heteroduplex forms a substrate for RNase H, an enzyme that recognizes and selectively destroys the RNA portion of the mRNA-ODN hybrid.

fact, the hybridization between antisense oligos and the target sequence, which has a particular three-dimensional structure resulting from secondary and tertiary structures, depends on the accessibility of the target sequence. Only small stretches of mRNA sequence, devoid of interchain hybridization, are available for heteroduplex formation with DNA oligonucleotides which affects the activity of ODNs. For example, only one of 34 ODNs targeting human c-raf mRNA demonstrated potent antisense activity. Modeling of the secondary structure of the target mRNA by computer software can be used for target selection of antisense molecules. Such a method carefully considers the potential folding pattern of a chosen mRNA as derived from its particular nucleotide sequence. After determining the free energy of a given secondary structure, the most probable folding structures are indicated, showing open loops and bulges that are accessible for oligonucleotides for efficient hybridization (Stein and Krieg, 1994; Monia, 1997; Wagner and Flanagan, 1997; Agrawal and Zhao, 1998).

Selection of chemical modifications

Cells contain a variety of exo and endonucleases that can degrade ODNs. A number of nucleotide and nucleoside modifications have made the oligonucleotide more resistant to nuclease digestion than the native ODNs that have phosphodiester linkages in their nucleotide backbone. Oligonucleotides that have been modified to enhance their nuclease resistance survive intact for longer times than unmodified oligonucleotides. A variety of oligonucleotide modifications have enhanced or conferred nuclease resistance, thus allowing oligos to reach their intracellular targets (Capaccioli et al., 1993; Gewirtz, 1993; Agrawal and Iyer, 1995; Galderisi et al., 1999). Phosphorothioates are one of the most frequent variants of ODNs. One of the oxygens in the phosphate backbone in these molecules is replaced by a sulfur atom (Fig. 2). Increased protection against cleavage by both exonucleases and endonucleases is the result of such chemical modification. Other modifications give rise to methylphosphonates, in which a methyl group is substituted for an oxygen of phosphate; other modifications are phosphoroamidates that show an amide linkage inserted instead of an ester bridge; peptide nucleic acid, having the phosphate sugar backbone substituted by an alkylamide linkage (Fig. 2; Eckstein, 1983; Henry et al., 1997b; Flanagan, 1998; Galderisi et al., 1999). The oligonucleotides also may be "chimeric oligonucleotides." Chimeric oligonucleotides contain two or more chemically distinct regions. These molecules are designed to confer more than one beneficial property to ODNs, such as increased nuclease resistance, increased uptake into cells, or increased binding affinity for the RNA target. At present, phosphorothioate oligos are the most widely used molecules in cell cultures, animals, and humans (Agrawal and Iyer, 1995; Monia, 1997; Agrawal and Zhao, 1997; Shinozuka et al., 1997; Galderisi et al., 1999).

Cellular delivery of ODNs

The main problem in increasing the bioavailability of administered ODNs is the protection against cleavage. While the mechanism involved in the cellular ODN uptake still is not clear, there also is a great variation between different cell types with regard to their ability to internalize oligo molecules. A receptor-mediated endocytosis seems to play a main role in ODN uptake, followed by the release of ODNs from endocytotic vesicles into the cytoplasm (Loke et al., 1989; Iversen et al., 1992; Bennett et al., 1994; Beltinger et al., 1995; Kronenwett and Haas, 1998b).

The cellular internalization of ODNs is not efficient in in vitro models, hence, many techniques have been used to enhance ODN uptake. The most widely used method is based on cationic lipids. These molecules form complexes with the anionic nucleic acids and protect them against degradation. The macromolecular complexes have a positive charge at the surface, allowing binding to cell membrane, which is negatively charged. Following attachment to the membrane, the complexes are taken up via endocytosis. Additional improvement in oligo uptake can be anticipated. For example, efforts are being made to modify liposomal lipids by adding ligands of cellular receptors as well as antibodies directed against antigens expressed on the respective target cells (Bennet et al., 1992; Capaccioli et al., 1993; Stein and Krieg, 1994; Beltinger et al., 1995; Gokhale et al., 1997; Kronenwett and Haas, 1998b).

While direct administration of ODNs in vitro is not an effective delivery method, phosphorothioate ODNs

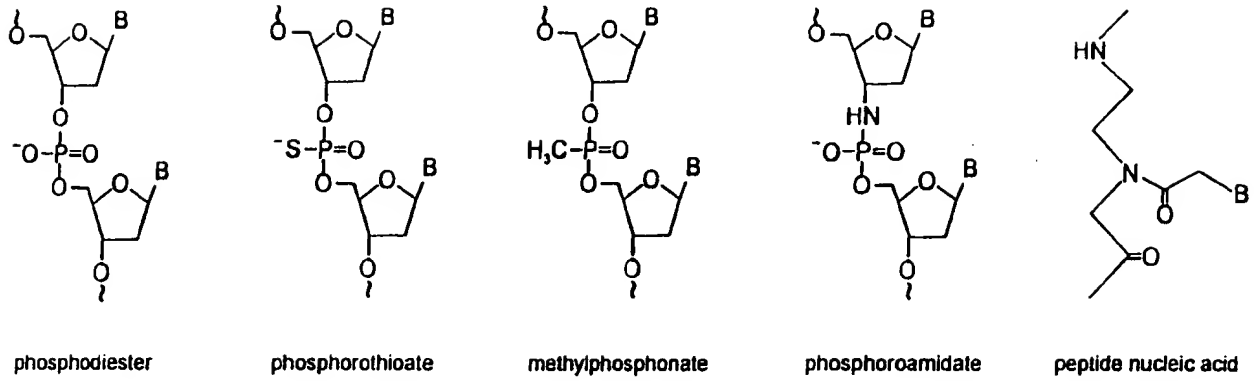

Fig. 2. Chemical structure of internucleotide linkages of unmodified (phosphodiester) and modified oligonucleotides. B, nucleotide bases.

administered intravenously without any delivery reagent to animal models showed effective and specific antisense inhibition. These surprising results helped revive antisense technology and encouraged researchers to move to clinical trials (Wagner, 1995; Crooke and Bennet, 1996; Geary et al., 1997).

Antisense oligonucleotide evaluation

The aim of antisense researchers is to show downregulation of a target gene in a sequence-specific manner, while control ODNs, which are oligonucleotides not complementary to the chosen target mRNA, should show little or no downregulation capability. However, several examples of nonsequence-specific effects have been seen with ODNs, particularly with the chemically modified molecules. Oligonucleotides, which are negatively charged, can interact with positively charged molecules. For example, ODNs can bind in a sequence-independent manner the gp120 protein of the human immunodeficiency virus-1 (HIV-1), bovine serum albumin, the receptor for platelet-derived growth factor, the receptor for basic fibroblast growth factor, and several other cellular proteins (Stein and Cheng, 1993; Stein and Krieg, 1994; Stein, 1995; Crooke and Bennet, 1996).

Antisense side effects also could be due to sequence-specific interactions between ODNs and cellular proteins that can cause the so-called "sequence-dependent but nonantisense effect." For example, the presence of four contiguous guanosine residues in an ODN, the G quartet, can result in an antiproliferative effect regardless of the remaining sequence of the molecule (Crooke and Bennet, 1996; Stein and Krieg, 1994; Vaerman et al., 1995; Wagner, 1995).

ANTISENSE THERAPY

The idea of antisense-mediated gene inhibition therapy is as fascinating as other types of gene therapy (Romano et al., 1998a; Giordano et al., 1998). The following examples suggest that these compounds may have some therapeutic efficacy likely through a combination of antisense and nonsequence-dependent effects on gene function.

Pharmacokinetics

Several experiments assessing the pharmacokinetics and toxicology of ODNs have been performed in mice, rats, and monkeys (Cossum et al., 1993; Galbraith et al., 1994; Iversen et al., 1995; Zhang et al., 1995; Leeds et al., 1998). The pharmacokinetics were independent of the length as well as of the sequence of ODNs. When injected intravenously or intraperitoneally, the nucleic acids were excreted mainly in the urine within 24 h. However, detectable levels were found in most tissues except the brain for up to 48 h, with only 15–50% degradation for phosphorothioate ODNs . In monkeys and in phase I clinical trials, dose-dependent hypotension, complement activation, and transient prolongation of thromboplastin time were observed as side effects. Preliminary results of other clinical studies demonstrated the safety and, to some extent, the efficacy of antisense ODNs in patients with malignant diseases (Iversen et al., 1995; Glover et al., 1997; Henry et al., 1997a; Raynaud et al., 1997; Sereni et al., 1999).

Antisense clinical trials for cancer treatment

A major signal transduction pathway involving the enzyme protein kinase C (PKC) has a critical influence on cell proliferation and differentiation (Liu and Heckman, 1998). An increased expression of PKC-alpha is found in many human cancers including those of the breast and colon and in brain tumors. Inhibition of human PKC-alpha gene expression has occurred with antisense ODNs both in vitro and in vivo (Dean et al., 1996; Zhang et al., 1997). A phosphorothioate ODN, directed against the 3′-untranslated region of PKC-alpha, has been tested by ISIS Pharmaceutical (Carlsbad, CA) and Novartis (Basel, Switzerland) in some human tumor cell lines grown in athymic mice. This oligo, named ISIS3521, was administered intravenously once a day for 14 days and showed a noticeable tumor growth decrease in T-24 bladder carcinoma, in A-549 non-small cell lung carcinoma, and in Colo 205 colon carcinoma xenograft models with a 50% inhibitory dose between 60 and 600 μg/kg per day. After this success, ODN entered a phase I clinical trial. Clinical responses were observed in 3 of 17 treated patients, all having ovarian cancer (McGraw et al., 1997; Flanagan, 1998).

The c-raf gene codes for a highly conserved serine-threonine-specific protein kinase (Magnuson et al., 1994; Kerkhoff and Rapp, 1998; Yuryev and Wennagle, 1998). Certain abnormal proliferative conditions are associated with raf expression and therefore are believed to be responsive to inhibition of raf expression (Worland et al., 1990). Examples of abnormal proliferative conditions are hyperproliferative disorders such as cancers, hyperplasias, pulmonary fibrosis, and angiogenesis (Nakatsu et al., 1986; Pfeifer et al., 1989; Naumann et al., 1997).

A phosphorothioate ODN named ISIS5132, which is complementary to c-raf mRNA, has shown a strong sequence-specific inhibition of c-raf gene expression in some subcutaneously implanted human tumor cell lines in nude mice. Subsequently, phase I clinical trials have demonstrated the safety of this ODN. Furthermore, several patients with breast, prostate, and colon cancers showed promising clinical response. Based on this data, phase II clinical trials were initiated in 1998 (Monia et al., 1996; Henry et al., 1997b; McGraw et al., 1997; Monia, 1997; Flanagan, 1998; Monteith et al., 1998).

The ODNs may also be useful for ex vivo bone marrow purging, a method used for treatment of patients suffering from leukemias and lymphomas. Large amounts of bone marrow can be surgically extracted from patients and stored in vitro, while the patients receive conventional treatment. Following relapse, the patients can be rescued by reinfusion of their own bone marrow cells that have been "purged" of residual malignant cells employing ODNs targeted against altered gene expression associated with the leukemias and/or lymphomas (De Fabritiis et al., 1998).

Bcr/abl mRNA is the product of a neo-gene created by a reciprocal translocation involving the c-abl and the bcr genes. The expression of the bcr/abl oncogene is involved in the pathogenesis of chronic myelogenous leukemia (CML). Some clinical experience with bcr/abl targeted antisense ODNs in CML has been reported (Vaerman et al., 1995; Skorski et al., 1997; Kronenwett and Haas, 1998a). De Fabritiis and colleagues (1998) treated a patient with CML in an accelerated phase with autologous bone marrow transplantation. Before reinfusion, cells were purged in vitro with a 26-mer phosphorothioate ODN targeted against bcr/abl mRNA. The patient was reported to be in complete hematological remission (Gewirtz, 1993; De Fabritiis et al., 1998).

The expression of the bcl2 gene, which is involved in the apoptosis pathway, is overregulated in most non-Hodgkin lymphomas (Reed et al., 1990; Tsurusawa et al., 1998; Reed, 1998). Genta (San Diego, CA) has developed an antisense ODN targeted against bcl2 mRNA. This oligo results in a complete remission in nude mice inoculated with human follicular lymphoma cells. Based on these results, phase I trials have been initiated (Raynaud et al., 1997; Webb et al., 1997; Flanagan, 1998; Kronenwett and Haas, 1998b; Bloem and Lockhorst, 1999; Chaudhary et al., 1999).

Antisense ODN as potential drugs in other human diseases

Antisense therapy is emerging as a potential agent for the treatment of cardiovascular diseases such as restenosis after angioplasty, vascular bypass graft occlusion, and transplant coronary vasculopathy. The local transfer of antisense molecules into the vascular wall offers a promising alternative for the treatment of atherosclerosis-related diseases at the cellular and molecular levels. Blood vessels are among the easiest targets for this gene therapy technique because in such conditions as postangioplasty restenosis, only a transient inhibition of target gene expression is required (Shi et al., 1994; Laitinen and Yla-Herttuala, 1998).

Coronary balloon angioplasty is a procedure in which a catheter bearing an inflatable distal balloon is inserted into the arterial lumen and expanded. The method is used to open stenotic regions in vessels closed by arterial plaques and fatty deposits. This technique is used often for patients suffering from atherosclerosis. Smooth muscle cell (SMC) proliferation of the vascular wall is a normal response to several pathophysiological stimuli, including those associated with procedures for mechanically opening stenoses. If the proliferation is extensive, restenosis could follow the procedure. In particular, as many as 50% of the patients undergoing successful coronary angioplasty can develop recurrent coronary artery obstructions. Several different classes of pharmacological agents have been employed to inhibit SMC proliferation but as yet, unsuccessfully.

One approach is to inhibit mitogens that act on the cell surface of SMCs. The c-myc gene product is encoded by an immediate-early response gene, the expression of which can be induced by various mitogens. C-myc expression is involved in the signal transduction pathways leading to cell division. Studies have demonstrated that proliferating cells have higher levels of c-myc mRNA and protein than do quiescent cells (Paggi et al., 1996; Nesbit et al., 1999).

Several investigators have demonstrated the in vitro growth-inhibitory effect of antisense oligomers targeting the c-myc proto-oncogene in human SMCs (Bennett et al., 1994; Shi et al., 1994). These in vitro studies provided the rationale for assessing c-myc antisense oligomers in the prevention of neointima in vivo. For this purpose, antisense oligomers targeted against c-myc mRNA were delivered into balloon-denuded porcine coronary arteries. Despite rapid plasma clearance following local delivery, oligomers persisted at the site of injection for at least 3 days, exceeding by severalfold their concentration in peripheral organs. The morphometric analyses, carried out 1 month after transcatheter c-myc antisense oligomer administration, showed a significant reduction in maximal neointimal thickness in the antisense-treated group compared with controls. These changes in vascular remodeling following denuding injury resulted in an increase in the residual lumen in the antisense-treated animals. Since c-myc antisense oligomers reduced the formation of neointima in denuded coronary arteries, a potential therapeutic use for the prevention of coronary restenosis can be hypothesized (Shi et al., 1994; Mannion et al., 1998).

The use of antisense oligonucleotides has also emerged as a powerful new approach as antiviral agents (Selvam et al., 1996; Caselmann et al., 1997; Lima et al., 1997; Wagner and Flanagan, 1997; Veal et al., 1998). In fact, the initial therapeutic applications of ODN were supposed to be as an antiviral agent. Ste-

phenson and Zamecnik (1978) disclosed antisense oligonucleotides as inhibitors of Rous sarcoma virus replication in chicken fibroblasts.

HIV is responsible for the disease that has come to be known as acquired immune deficiency syndrome (AIDS). The HIV genome tends to mutate at a high rate, causing great genetic variation between strains of the virus and between virus particles of a single infected individual. Therapeutic agents currently used in the treatment of AIDS often cause severe side effects that preclude their use in many patients (Zhang et al., 1995; Junker et al., 1997; Romano et al., 1998b; Sereni et al., 1999).

One method for inhibiting specific gene expression that is believed to have promise is the antisense approach. Inhibition of viral gene expression and replication can be efficiently achieved by targeting the conserved sites of the viral RNAs that signal the synthesis of conserved HIV proteins, particularly the p24 core antigen protein. Some research groups have synthesized 20 mer/15 mer sequences targeted against the p24 core protein region of HIV. Initial clinical trials are based on ODN systemic administration (intravenously). Dosages that can be used for systemic administration preferably range from about 0.01 to 50 mg/kg administered once or twice per day. Evaluations of ODN activity are under examination (Zhang et al., 1995; Junker et al., 1997; Sereni et al., 1999).

Chronic infection with the hepatitis B virus (HBV) is a major health problem worldwide. The only established treatment is interferon with an efficacy of only 30–40% in highly selected patients. The discovery of animal viruses closely related to the HBV has contributed to active research on antiviral therapy of chronic HBV infection. The animal model tested and described by several authors are Peking ducks infected with the duck HBV (DHBV; Shinozuka et al., 1997; Soni et al., 1998; Xin and Wang, 1998). Molecular therapeutic strategies are based on antisense ODNs directed against the 5′-region of the preS gene of DHBV that inhibited viral replication and gene expression in vitro in primary duck hepatocytes. The in vivo studies showed that intravenous injection of antisense ODNs entrapped within liposomes enhances delivery of the ODNs to the liver and inhibits DHBV replication. Serum DHBV DNA levels fall rapidly, with a corresponding decrease in intrahepatic viral replicative intermediates at the end of the 5-day study period. These results demonstrate a potential clinical use for antisense DNA as antiviral therapeutic agents (Caselmann et al., 1997; Lima et al., 1997; Offensperger et al., 1998; Soni et al., 1998).

THE FIRST ANTISENSE-BASED DRUG

Vitravene is the first in a class of novel therapeutic agents based on an antisense mechanism that has been approved for marketing in the United States. Vitravene is indicated for the local treatment of cytomegalovirus (CMV) retinitis in patients with AIDS who are intolerant to or have a contraindication to other treatments for CMV retinitis or who were insufficiently responsive to previous treatments (from www.vitravene.com). Vitravene consists of a phosphorothioate oligonucleotide designed to inhibit human CMV replication by an antisense mechanism. It has been shown in vitro to inhibit replication of human CMV with a greater potency than either ganciclovir or foscarnet. It does not interfere with the antiviral activity of the anti-HIV-drugs AZT and dideoxycitidine and it can be additive to the use of ganciclovir and foscarnet. Vitravene was equally potent against 21 independent clinical human CMV isolates, including several that were resistant to ganciclovir, foscarnet, and/or cidofovir (from www.vitravene.com).

FUTURE PROSPECTS

The use of antisense to modify gene expression is variable in both its efficacy and reliability, which caused objections about its use as a therapeutic agent. Most of these concerns can be overcome by the development of a new generation of antisense molecules with improved target specificity and enhanced delivery to the target cells. However, one concept must be borne in mind, an oligonucleotide need not be exclusively complementary to its target nucleic acid sequence to be specific. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule sufficient to cause a loss of function. There is a sufficient degree of complementarity to avoid nonspecific binding of the oligonucleotide to nontarget sequences (Stein and Krieg, 1994; Stein, 1995; Monia, 1997).

Antisense ODNs already have shown their effectiveness in several preclinical studies. Phosphorothioate ODNs have reached phase I and II in clinical trials for the treatment of cancer and viral infections and have demonstrated an acceptable safety and pharmacokinetic profile for continuing their development. The new drug Vitravene, which is based on an antisense mechanism and is commercially available in the United States, has shown that some substantial successes can be reached with the antisense technique.

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Targeting of antisense DNA: comparison of activity of anti-rabbit beta-globin oligodeoxyribonucleoside phosphorothioates with computer predictions of mRNA folding.

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To assess the usefulness of computer-assisted modeling of mRNA as an aid in design of antisense DNA, the efficiency of inhibition of translation of rabbit beta-globin mRNA by various antisense sequences was compared with calculated structures of the mRNA. The model obtained by consideration of 30 lowest-energy computer-simulated structures is consistent with the high accessibility of the AUG initiation codon region known from digestion with nucleases and with previous antisense inhibition studies reported in the literature. Additional antisense inhibition data were obtained with 20-mer phosphorothioate oligonucleotides, targeted to regions of beta-globin mRNA differing moderately in their degree of participation in intramolecular folding. The efficiency of translation arrest by the oligonucleotides in cell-free expression systems (wheat germ extract and rabbit reticulocyte lysate) was obtained by measuring incorporation of [35S]methionine into total protein, and corrected for sequence-nonspecific inhibition using brome mosaic virus mRNA. In the presence of RNase H (wheat germ system), the inhibitory activity of the oligonucleotides showed correlation with the calculated secondary structure of mRNA, in particular at low oligonucleotide-to-mRNA ratios (correlation coefficient, 0.95). No correlation was observed in the reticulocyte lysate system, in which the inhibition is mediated by translational arrest.

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